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Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain α-keto acid dehydrogenase

Kun Zhu,[1] Darrell O. Bayles,[2] Anming Xiong,[1] R. K. Jayaswal[1] and Brian J. Wilkinson[1]

Correspondence
Brian J. Wilkinson
bjwilkin@ilstu.edu

[1]Microbiology Group, Department of Biological Sciences, Illinois State University, Normal, IL 61790-4120, USA

[2]Microbial Food Safety Research Unit, Eastern Regional Research Center (ERRC), Agricultural Research Service, US Department of Agriculture, Wyndmoor, PA 19038, USA

Branched-chain fatty acids (BCFAs) typically constitute more than 90 % of the fatty acids of *Listeria monocytogenes*. The authors have previously described two Tn*917*-induced, cold-sensitive, BCFA-deficient (< 40 %) *L. monocytogenes* mutants (*cld-1* and *cld-2*) with lowered membrane fluidity. Sequence analyses revealed that Tn*917* was inserted into different genes of the branched-chain α-keto acid dehydrogenase cluster (*bkd*) in these two mutants. The cold-sensitivity and BCFA deficiency of *cld-1*, in which Tn*917* was inserted into *bkdB*, were complemented *in trans* by cloned *bkdB*. The growth and corresponding BCFA content of the mutants at 37 °C were stimulated by fatty acid precursors bypassing Bkd, 2-methylbutyrate (precursor for odd-numbered anteiso-fatty acids), isobutyrate (precursor for even-numbered iso-fatty acids) and isovalerate (precursor for odd-numbered iso-fatty acids). In contrast, the corresponding Bkd substrates, α-ketomethylvalerate, α-ketoisovalerate and α-ketoisocaproate, exhibited much poorer activity. At 26 °C, 2-methylbutyrate and isovalerate stimulated the growth of the mutants, and at 10 °C, only 2-methylbutyrate stimulated growth. Pyruvate depressed the BCFA content of *cld-2* from 33 % to 27 %, which may be close to the minimum BCFA requirement for *L. monocytogenes*. The transcription of *bkd* was enhanced by Bkd substrates, but not by low temperature. When provided with the BCFA precursors, *cld-2* was able to increase its anteiso-$C_{15:0}$ fatty acid content at 10 °C compared to 37 °C, which is the characteristic response of *L. monocytogenes* to low temperature. This implies that Bkd is not the major cold-regulation point of BCFA synthesis.

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INTRODUCTION

Listeria monocytogenes causes the potentially life-threatening infection known as listeriosis, which has a fatality rate of 20–25 % (Mead *et al.*, 1999). Listeriosis outbreaks have been linked to the consumption of food contaminated with this organism. Nowadays, there is an increased consumption of ready-to-eat, chilled and frozen foods, with decreased use of chemical microbial control agents. The ability of *L. monocytogenes* to grow at refrigeration temperatures is a critical aspect of its role as a food-borne pathogen, since refrigeration temperature inhibits the growth of most other food-borne pathogens (Bryan, 2004). However, the underlying psychrotolerance mechanism is not completely understood, and a better understanding may result in a novel strategy for controlling the growth of *L. monocytogenes* at low temperatures.

Low temperature has profound effects on all aspects of microbial cell structure and function, involving the structural integrity of macromolecules, macromolecular assemblies, protein synthesis and nutrient uptake (Panoff *et al.*, 1998; Weber & Marahiel, 2002). Small, nucleic-acid-binding, cold-shock proteins play key roles in resuming growth under cold shock (Panoff *et al.*, 1998). Low

Abbreviations: BCFA, branched-chain fatty acid; Bkd, branched-chain α-keto acid dehydrogenase; IB, isobutyrate; IV, isovalerate; KIC, α-ketoisocaproate; KIV, α-ketoisovalerate; KMV, α-ketomethylvalerate; MB, 2-methylbutyrate; PDH, pyruvate dehydrogenase.

The GenBank accession number for the sequence reported in this paper is AY138856.

temperature also reduces membrane fluidity, and causes membrane phase transitions from a liquid-crystalline state to a more rigid gel-like state. To restore membrane functionality at low temperatures, fatty acids with low melting points are incorporated into lipids. The two most common ways bacteria increase membrane fluidity are by incorporating unsaturated and branched-chain fatty acids (BCFAs), which have lower melting points than the corresponding saturated straight-chain fatty acids, into their lipids (Suutari & Laakso, 1994). The *L. monocytogenes* membrane-fatty-acid composition is dominated to an unusual extent by BCFAs (>90 % of the total fatty acid content) (Annous *et al.*, 1997). Among the common BCFAs in *L. monocytogenes*, anteiso-$C_{15:0}$ has the lowest melting point (Kaneda, 1991). When the growth temperature declines, anteiso-$C_{15:0}$ content in the membrane rises to maintain optimal membrane fluidity (Annous *et al.*, 1997; Edgcomb *et al.*, 2000; Nichols *et al.*, 2002).

Two Tn*917* transposon-induced mutants (*cld-1* and *cld-2*) have lost the ability to grow at 4 °C on solid media, but are not defective in the induction of cold-shock proteins (Bayles *et al.*, 1996). These mutants are deficient in the production of odd-numbered BCFAs, and exhibit atypical amounts of even-numbered straight-chain and iso-BCFAs (Annous *et al.*, 1997; Edgcomb *et al.*, 2000). The membranes of strain *cld-1* are significantly less fluid than those of the parent strain (Edgcomb *et al.*, 2000; Jones *et al.*, 2002). To further characterize this mechanism of psychrotolerance, it was important to identify the disrupted gene(s) that caused the cold-sensitive phenotype in the mutants. Based on the observations above, we speculated that the insertion of Tn*917* into *cld-1* and *cld-2* may interrupt the gene(s) involved in BCFA biosynthesis (Annous *et al.*, 1997). In bacteria, the branched-chain portion of fatty acids derives primarily from the branched-chain amino acids (Kaneda, 1991; de Mendoza *et al.*, 2002). Moreover, incorporation of a methyl branch can only be achieved by *de novo* biosynthesis of fatty acid (Suutari & Laakso, 1994). Previous studies of BCFA biosynthesis have primarily focused on *Bacillus subtilis* (de Mendoza *et al.*, 2002). Two critical enzymes in the pathway from branched-chain amino acids to BCFA have been reported: branched-chain α-keto acid dehydrogenase (Bkd) and β-ketoacyl-acyl carrier protein synthase III (FabH) (Oku & Kaneda, 1988; Choi *et al.*, 2000; Lu *et al.*, 2004). Isoleucine, valine and leucine are transaminated by branched-chain amino acid transaminase, and are subsequently oxidatively decarboxylated by the Bkd complex, resulting in production of the short branched-chain acyl-CoA derivatives 2-methylbutyryl-CoA, isobutyryl-CoA and isovaleryl-CoA (Fig. 1). These acyl-CoA precursors are then utilized by FabH as starting units to initiate BCFA

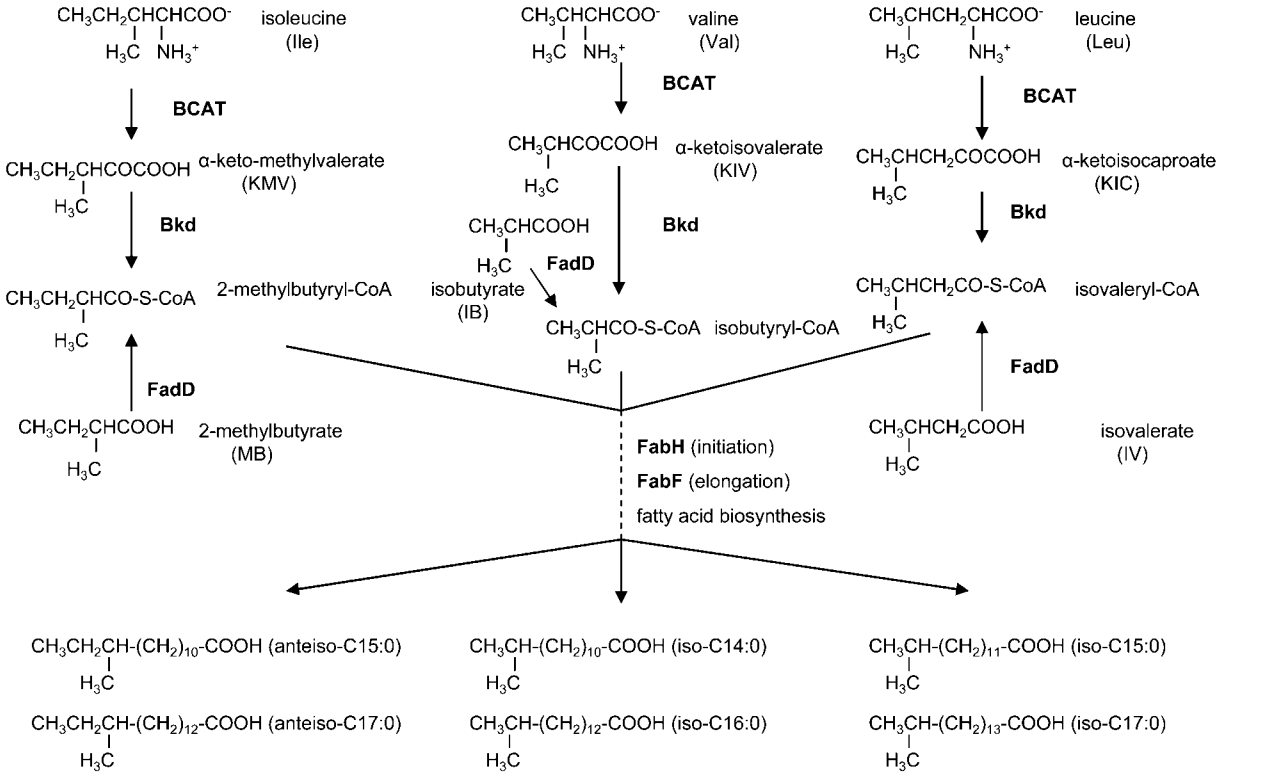

Fig. 1. Schematic biosynthesis pathway of starting units for BCFAs from branched-chain amino acids in *L. monocytogenes*. BCAT, branched-chain amino acid transaminase; Bkd, branched-chain α-keto acid dehydrogenase; FabH, β-ketoacyl-ACP synthase III; FadD, acyl-CoA synthetase; FabF, β-ketoacyl ACP synthase II.

biosynthesis (Fig. 1). Although the cold-regulated fatty acid adjustment is one universal adaptation to low temperature, the underlying mechanism of cold-regulated BCFA synthesis is not well characterized (de Mendoza *et al.*, 2002; Lu *et al.*, 2004) Here, we identified that the two *L. monocytogenes* cold-sensitive mutants (*cld-1* and *cld-2*) had Tn*917* inserted into the *bkd* cluster. We also attempted to determine the role of Bkd in biosynthesis and cold-regulation of BCFA.

METHODS

Bacterial strains and growth conditions. *L. monocytogenes* parent strain 10403S, and transposon-insertion mutants *cld-1* and *cld-2*, were used in this study (Annous *et al.*, 1997; Bayles *et al.*, 1996). These strains were grown in BHI (Difco) supplemented with erythromycin (1 μg ml^{-1}) and lincomycin (25 μg ml^{-1}) for strains *cld-1* and *cld-2*, at 37 °C with shaking (150 r.p.m.), unless specified otherwise. *Escherichia coli* strain Top10 (Invitrogen) was grown in Luria–Bertani (LB) medium at 37 °C with shaking (220 r.p.m.). When necessary, ampicillin, kanamycin and tetracycline were added to final concentrations of 100, 20 and 10 μg ml^{-1}, respectively. For preparation of *L. monocytogenes* cultures for growth studies and fatty acid analysis, the 5 ml starter culture without supplements was prepared as described above. For all strains, a 2 ml volume of the overnight starter culture was used to inoculate 100 ml BHI medium without antibiotics in a 250 ml Erlenmeyer flask. All supplements, short BCFAs, branched-chain α-keto acids and pyruvate were added to the medium as filter-sterilized solutions. Growth was monitored by measuring OD_{600} using a Beckman DU-65 spectrophotometer.

Molecular biological methods. Unless specified, all molecular biological methods were performed as described by Sambrook *et al.* (1989). Chromosomal DNA from *L. monocytogenes* was isolated using a Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's manual, except that cells were lysed by lysozyme (20 μg ml^{-1}) at 37 °C for 60 min.

Cloning and sequencing the *L. monocytogenes* DNA adjacent to the transposon insertions. The *L. monocytogenes* mutants in this study carry a Tn*917* insertion (Camilli *et al.*, 1990). Genomic DNA was digested with restriction enzyme *Eco*RI, self-ligated and transformed into *E. coli* Top10 (Invitrogen). Plasmid DNA was isolated from kanamycin-resistant transformants using a miniprep kit (Qiagen). Chromosomal DNA in the resultant plasmid was sequenced using the Dye Terminator Cycle Sequencing Kit and an ABI Prism 310 sequencer (Applied Biosystems). The sequencing reaction was performed using two oligonucleotides 5′-GGAGCATATCACTTTTCTTGGAGAG-3′ and 5′-ACGGTTGAAAACTGTACC-3′, respectively corresponding to the 5′ and 3′ ends of Tn*917*. Using oligonuleotides 5′-AGATGTTGGGAAAAAAGGTGGC-3′ and 5′-CTTGAAGAATAGCGGCTTGTGG-3′, the DNA region that spanned the portion of the genome between the two Tn*917* insertion sites was cloned into the PCR cloning vector pCR2.1 (Invitrogen), and subsequently sequenced using primer walking (Fig. 2). The inverse PCR procedure with oligonuleotides 5′-GAAACAATGCTAATGGCGAG and 5′-CTCCAATCGCATAGATGTG-3′ was used to amplify the DNA segment beyond the *Eco*RI site flanking the Tn*917* insertion site. Nucleic acid and deduced amino acid sequence data were analysed using MacVector 6.5 edition (Oxford Molecular) and the National Center for Biotechnology Information (NCBI) BLAST network service.

Complementation of mutant *cld-1* with intact *bkdB*. The wild-type *bkdB* gene was amplified using primers pE25 (5′-AAAGGATCCATGCCCAAATTAGGGGAA-3′) and pE23 (5′-GAGGAGTTTTCTTCCGCAATGA-3′). The putative promoter region of the *bkd* cluster was amplified using primers pMO52 (5′-CACCAAGGTATTACATCAATTA-3′) and pMO32 (5′-TTCGGATCCATCTGAAAAAAATCTGCTT-3′) (Glaser *et al.*, 2001) (Fig. 2). The two fragments were cloned into *Hin*dIII and *Pst*I double-digested pKSV7 (Smith & Youngman, 1992), giving rise to pME2K8. The *Bam*HI restriction sites in primer pMO32 and pE25 were used to fuse the promoter region with *bkdB* into a correct reading frame. A tetracycline resistance gene was subsequently cloned into the *Hin*dIII site of pME2K8 to generate pME2K8tet. The electroporation was performed essentially according to the method of Park & Stewart (1990). The transformants were plated on BHI agar containing tetracycline, and incubated at 30 °C for 2–3 days.

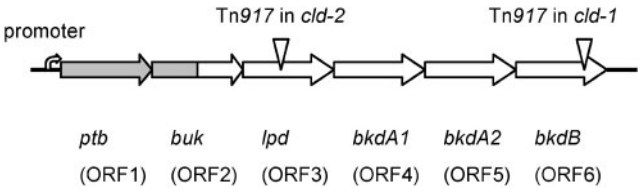

Fig. 2. Molecular organization of the *L. monocytogenes bkd* gene cluster and transposon insertion sites. The DNA sequence of the grey portion was from the *L. monocytogenes* genome sequence (Glaser *et al.*, 2001).

RNA isolation and Northern blot analysis. In the branched-chain α-keto acid induction analysis, strain 10403S was grown in BHI medium supplemented with 500 μM α-ketomethylvalerate at 37 °C. In the cold-induction analysis, strain 10403S was grown in separate BHI cultures at 37 °C and 10 °C. When the OD_{600} reached 0·3–0·4, cells were chilled on ice, and harvested by centrifugation. RNA protection solution (Qiagen) was added to stabilize RNA immediately after harvesting. A 1 ml volume of RLT buffer (RNeasy Mini Kit, Qiagen) and 0·5 g glass beads were added to the cell pellet obtained from 2·5 ml of culture. A bead beater (Biospec Products) was used to break the cells (5000 r.p.m., 20 s, six times). Total RNA was isolated using an RNeasy Mini Kit according to the manufacturer's instructions. RNA was treated with DNase I (Invitrogen) prior to Northern blot analysis. Northern hybridization was performed using the NorthernMax kit according to the manufacturer's manual (Ambion).

Fatty acid analysis. Cultures of *L. monocytogenes* were grown in 100 ml BHI medium at designated temperatures in the presence or absence of the following supplements: short BCFAs bypassing Bkd [2-methylbutyrate (MB), isobutyrate (IB) and isovalerate (IV)], substrates of Bkd [α-ketomethylvalerate (KMV), α-ketoisovalerate (KIV) and α-ketoisocaproate (KIC)] and pyruvate. Cells were harvested in the mid-exponential phase (OD_{600}, 0·5–0·7), and the pellet was washed three times with distilled water. The fatty acids in the cells (30–40 mg wet weight) were saponified, methylated and extracted. The resulting methyl ester mixtures were separated by an Agilent 5890 dual-tower gas chromatograph. Fatty acids were identified by the MIDI microbial identification system (Sherlock 4.5 microbial identification system). This analysis was performed at Microbial ID (Newark, DE, USA).

RESULTS

Tn*917* was inserted in the *bkd* region in *cld-1* and *cld-2*

BLAST search of the sequences flanking transposon insertion sites revealed that in *cld-1* and *cld-2*, the transposon

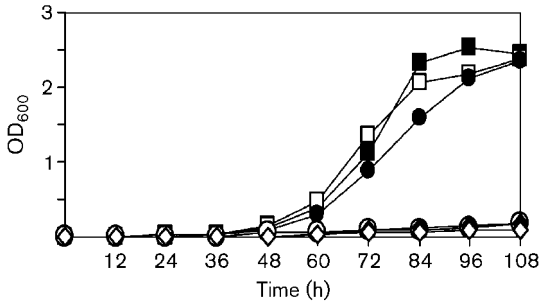

Fig. 3. Growth of *L. monocytogenes* 10403S, *cld-1*, *cld-2*, and their *bkdB*-complemented strains in BHI at 10 °C. □, 10403S; ■, 10403S-*bkdB*; ○, *cld-1*; ●, *cld-1-bkdB*; ◇, *cld-2*; ◆, *cld-2-bkd*.

appeared to insert in the same gene cluster encoding the Bkd complex. In *B. subtilis*, which is a close relative of *L. monocytogenes*, Bkd is a multi-enzyme complex encoded by four genes (Wang *et al.*, 1993); therefore a genome segment of 5600 bp flanking the insertion sites was cloned and sequenced in order to further characterize the *bkd* cluster. Four complete ORFs (ORF3, 4, 5 and 6) and one incomplete ORF (ORF2) were found in this segment (Fig. 2), all with the same transcription orientation. Deduced amino acid sequences of the four complete ORFs were most similar (35–82 % identity) to the subunits of the prokaryotic Bkd complex, including those from *B. subtilis* and *Staphylococcus aureus*. ORF3 (*lpd*), ORF4 (*bkdA1*), ORF5 (*bkdA2*) and ORF6 (*bkdB*) respectively encoded dihydrolipoamide dehydrogenase (E3), decarboxylase (E1α), decarboxylase (E1β) and dihydrolipoamide acyltransferase (E2) subunits of the Bkd complex. Specifically, Tn*917* was located near the 3′ end of *bkdB* in strain *cld-1*, and in the middle of *lpd* in strain *cld-2* (Fig. 2). The organization of these genes was identical to the order in *B. subtilis* and *Enterococcus faecalis* (Debarbouille *et al.*, 1999; Ward *et al.*, 1999). In addition, a comparison of several *L. monocytogenes* genomic sequences revealed a BCFA kinase gene (*buk*, ORF2) and a branched-chain phosphotransacylase gene (*ptb*, ORF1) upstream of *lpd* (Fig. 2) (Glaser *et al.*, 2001; Nelson *et al.*, 2004), which were also present in the *bkd* operon of *B. subtilis* and *E. faecalis* (Debarbouille *et al.*, 1999; Ward *et al.*, 1999).

Genetic complementation restores the growth of *cld-1* and its BCFA content

In order to confirm that the transposon insertion in the mutants was the cause of the cold-sensitive and BCFA-deficient phenotype, the mutation was complemented by a plasmid bearing the wild-type *bkdB* gene. Shuttle vector pME2K8tet contained the putative promoter region and the complete *bkdB* gene (Fig. 2). Transformant *cld-1-bkdB* grew normally on BHI agar at 37 °C, as did strain 10403S. Furthermore, *cld-1-bkdB* showed the same growth pattern as 10403S at 10 °C (Fig. 3). As shown in Table 1, functional *bkdB* also restored the normal BCFA content in *cld-1* from 39 % to 98 % of total fatty acids. Clearly the BCFAs were closely associated with the growth ability of *L. monocytogenes* at low temperature. These data demonstrate that *bkdB* is important for the BCFA synthesis, and that the interrupted *bkdB* in strain *cld-1*causes the BCFA deficiency. As a negative control, the intact *bkdB* did not fully restore the BCFA and growth of *cld-2*, presumably because of a lack of a wild-type *lpd* gene in the pME2K8tet construct.

BCFA precursors bypassing Bkd stimulate the growth and BCFA content of *cld-1* and *cld-2*

Strains *cld-1* and *cld-2* grew very poorly at 10 °C, and they grew more slowly than parent strain 10403S at 37 °C (Fig. 4). It was expected that BCFA precursors bypassing Bkd, i.e. the short BCFAs MB, IB and IV, would restore the growth and BCFAs of the *bkd* mutants if the primary role of Bkd in *L. monocytogenes* is to produce BCFA precursors (Fig. 1). We previously reported that MB stimulated the growth of *cld-1* and *cld-2* (Annous *et al.*, 1997), and in this study we found that IB and IV could also stimulate the growth of these strains at 37 °C (Fig. 4a). Here and subsequently, growth and fatty acid data are shown for *cld-2* only. The growth and fatty acid data for *cld-1* were very similar, but the growth of *cld-2* was more impaired, and the BCFA

Table 1. Fatty acid composition of 10403S, *cld-1*, *cld-2* and their *bkdB*-complemented strains in BHI at 37 °C

The values are the means of at least two independent experiments ±SEM; some minor fatty acid components are not shown. ND, Not detected.

Strain	Percentage (w/w) of total fatty acids									
	$C_{14:0}$	Iso-$C_{14:0}$	$C_{15:0}$	Iso-$C_{15:0}$	Anteiso-$C_{15:0}$	$C_{16:0}$	Iso-$C_{16:0}$	Iso-$C_{16:0}$	Anteiso-$C_{17:0}$	BCFA
10403S	ND	0·7±0·1	ND	10·4±0·4	46·2±0·6	0·9±0·1	2·5±0·1	3·4±0·2	35·3±0·4	98·7
10403S-*bkdB*	ND	0·7±0·0	ND	10·5±0·3	46·2±0·2	1·2±0·2	2·6±0·0	3·5±0·1	35·0±0·3	98·5
cld-1	18·1±1·1	10·7±2	6·2±1·6	2·0±0·7	10·3±1·4	34·2±1·3	13·3±1·9	ND	3·5±1·5	39·8
cld-1-bkdB	ND	0·5±0·0	ND	10·1±0·4	47·0±0·4	0·9±0·1	2·2±0·1	3·4±0·1	35·6±0·2	98·9
cld-2	27·5±0·7	13·6±1·6	5·2±0·6	1·0±0·1	3·1±0·3	30·8±1·4	14·8±1·5	ND	0·9±0·0	33·6
cld-2-bkdB	17·5±0·8	16·0±4·4	4·3±0·5	1·4±0·0	5·1±1·4	32·4±6·4	18·6±2·4	ND	1·6±0·1	42·8

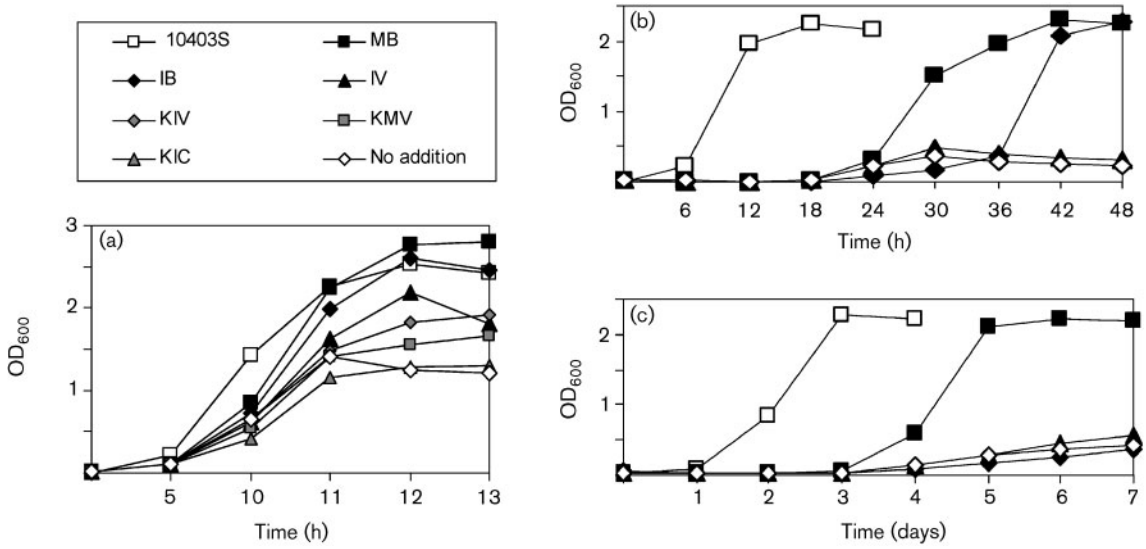

Fig. 4. Growth of *L. monocytogenes* strain *cld-2* in BHI with various supplements at 37 °C (a), 26 °C (b) and 10 °C (c). IV, IB and MB were added to a final concentration of 100 μM. KIC, KMV and KIV were added to a final concentration of 500 μM. *L. monocytogenes* 10403S was grown in BHI without supplement.

content was somewhat less than *cld-1*. IV showed less stimulatory activity than MB and IB. Supplementation of BHI medium with these precursors greatly increased the corresponding BCFAs. MB increased anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$, IB increased iso-$C_{14:0}$ and iso-$C_{16:0}$, and IV increased iso-$C_{15:0}$ and iso-$C_{17:0}$ (Table 2), whereas the straight-chain fatty acid precursors propionate and butyrate increased the corresponding straight-chain fatty acid content (data not shown). In contrast, under the same conditions, these precursors did not show any significant effect on the fatty acid composition of wild-type 10403S (data not shown). The overall total BCFA content was fully restored by MB and IB, and partially restored by IV, which was in accordance with the lower growth-stimulatory activity of IV compared to either MB or IB at 37 °C. IB greatly increased the even-numbered, iso-BCFAs in *cld-2* from 29 % to 88 %, and fully restored the growth. Moreover, increases of IV concentration further restored the odd-numbered, iso-BCFA content and growth (data not show). The IB- and IV-stimulated growth indicated that optimal growth at 37 °C could also be achieved by odd-numbered or even-numbered iso-BCFAs alone, without anteiso-BCFAs,

Table 2. Fatty acid composition of *cld-2* in BHI with various supplements at 37 °C

The values are the means of at least two independent experiments ±SEM; some minor fatty acid components are not shown. ND, Not detected.

Supplement*	Percentage (w/w) of total fatty acids									
	$C_{14:0}$	iso-$C_{14:0}$	$C_{15:0}$	Iso-$C_{15:0}$	Anteiso-$C_{15:0}$	$C_{16:0}$	Iso-$C_{16:0}$	Iso-$C_{17:0}$	Anteiso-$C_{17:0}$	BCFA
None	27·5±0·7	13·6±1·6	5·2±0·6	1·0±0·1	3·1±0·3	30·8±1·4	14·8±1·5	ND	0·9±0·0	33·6
MB	1·7±0·3	1·7±0·6	1·2±0·3	0·6±0·1	48·4±1·5	7·1±0·7	5·9±1·7	ND	33·1±1·9	89·8
IB	2·4±0·4	40·2±1·3	1·0±0·3	0·4±0·1	2·0±0·2	4·9±0·3	48·3±0·6	ND	ND	91·2
IV	17·8±5·3	17·4±7·6	3·8±0·3	13·6±3·2	2·8±0·5	21·6±5·3	18·5±7·7	1·8±0·3	0·7±0·2	55·0
KMV	27·7±0·9	7·5±0·3	4·4±0·2	1·3±0·1	12·9±0·5	27·2±0·5	10·6±0·7	ND	3·8±0·1	36·2
KIV	22·5±0·2	23·3±0·3	3·6±0·1	1·1±0·0	1·7±0·1	20·5±0·1	24·1±0·5	ND	0·4±0·0	50·7
KIC	28·8±2·3	9·6±1·6	5·5±0·4	6·3±0·3	2·3±0·3	31·6±1·5	10·5±1·9	0·9±0·1	0·6±0·1	30·4
MB+IB	1·6±0·1	29±0·8	0·5±0·0	0·3±0·0	16·9±2·4	3·0±0·1	44·4±2·1	ND	4·1±0·6	94·8
MB+IV	1·7±0·2	3·2±1·2	0·8±0·2	22·9±2·4	35·9±1·0	5·0±0·5	7·6±2·7	5·6±0·5	16·8±0·8	92·2
Pyruvate	30·7±0·4	10·2±0·7	5·5±0·1	1·4±0·1	2·6±0·1	35·4±1·7	11·7±1·0	ND	0·7±0·0	27·2

*Supplements were added to the following final concentrations: MB, IB and IV, 100 μM; KMV, KIV and KIC, 500 μM; MB+IB, 50 μM and 500 μM, respectively; MB+IV, 50 μM and 500 μM, respectively; pyruvate, 500 μM.

although anteiso-BCFAs are normally the most abundant BCFAs (>80 %) in *L. monocytogenes* (Table 1).

Substrates of Bkd do not fully restore the growth of mutants

To further confirm that the Bkd complex in *cld-1* and *cld-2* was not functioning properly, the effect of KMV, KIV and KIC, which are all substrates of Bkd, was investigated. In contrast to the short BCFAs described above, these substrates, at concentrations of 100 μM, did not show any apparent stimulatory effect on growth (data not shown). At concentrations of 500 μM, KMV and KIV resulted in better growth of the mutants at 37 °C, whereas KIC only slightly stimulated growth (Fig. 4a). The corresponding BCFAs were also increased to some extent by these substrates (Table 2). However, the overall activities of substrates were significantly less than those of short BCFAs, and iso precursor KIV showed higher activity than KMV and KIC, which was not the case with short BCFAs, where anteiso precursor MB was most active (Fig. 1, Table 2). The low activities of Bkd substrates demonstrated that the Bkd in *cld-1* and *cld-2* was not functioning efficiently, or perhaps not at all.

Temperature affects the growth-stimulatory activities of the BCFA precursors

As described above, addition of short BCFAs extensively changed the fatty acid composition in *bkd* mutant *cld-2* (Table 2). To clarify the influence of different fatty acid compositions on the growth of *L. monocytogenes* at low temperature, *cld-2* was grown at different temperatures in medium supplemented with these BCFA precursors. The stimulatory activities of the short BCFAs showed significant temperature dependence. At 26 °C, MB and IB restored the growth of *cld-2* to the same level as the wild-type, but there was a prolonged lag phase not observed with strain 10403S (Fig. 4b). IV exhibited no apparent growth-stimulatory activity at 26 °C. Only MB restored the growth of *cld-2* at 10 °C (Fig. 4c). Furthermore, the temperature-dependent activity prompted us to examine whether IB and IV could depress the stimulatory activity of MB at low temperature. When IB, IV and MB were added at the same concentrations, IB and IV exhibited little or no effect (data not shown). However, an excess of IB and IV significantly depressed the growth-stimulatory activity of MB at 10 °C (Fig. 5). IB produced a stronger depression than IV, which was consistent with the fatty acid data showing that IB caused a greater decrease in anteiso-$C_{15:0}$ (Table 2). In contrast, at 37 °C, the same excess of IB and IV did not depress the MB-stimulated growth, but stimulated growth to a level above that with MB alone (data not shown). These data demonstrate that although *L. monocytogenes* is able to achieve optimal growth at 37 °C when it has a high content of either anteiso-BCFAs or iso-BCFAs, there must be a substantial amount of anteiso-$C_{15:0}$ for growth at low temperatures.

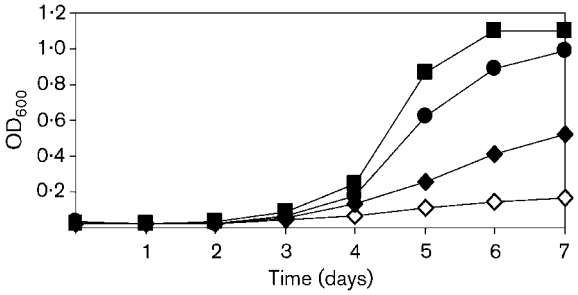

Fig. 5. Growth of *L. monocytogenes cld*-2 in BHI at 10 °C with mixed BCFA precursors. ◇, BHI without supplement; ■, 50 μM MB; ●, 50 μM MB plus 500 μM IV; ◆, 50 μM MB plus 500 μM IB.

Pyruvate further decreases the BCFA content in *cld-2*

The decreased, but not completely abolished, BCFA content of the *bkd* mutants indicated that the ability to produce BCFA was not completely lost, and implied that a compensatory pathway was operating (Table 2). Subunits of Bkd and pyruvate dehydrogenase (PDH) in *L. monocytogenes* showed considerable homology (36–48 % identity) (Glaser *et al.*, 2001), and therefore PDH in *cld-2* might metabolize branched-chain α-keto acids to short branched-chain acyl-CoA derivatives. This notion was supported by the observation that exogenous pyruvate further increased the straight-chain $C_{14:0}$ and $C_{16:0}$ fatty acids, and concomitantly decreased the BCFA content in *cld-2* from 33 % to 27 % (Table 2). However, exogenous pyruvate did not affect the fatty acid composition of 10403S (data not shown).

bkd transcription is induced by substrates, but not low temperatures

The role of the Bkd substrates in the regulation of *bkd* expression was investigated by Northern analysis. As shown in Fig. 6, a 7 kb transcript was detected, which might cover the whole *bkd* cluster, suggesting that *ptb*, *buk*, *lpd*, *bkdA1*, *bkdA2* and *bkdB* were co-transcribed as an operon. The 7 kb *bkd* transcript was induced by exogenous branched-chain α-keto acids (Fig. 6a), which was similar to *bkd* induction in *B. subtils* and *E. faecalis* (Debarbouille *et al.*, 1999; Ward *et al.*, 2000). Since cold-regulated BCFA synthesis is one of the major adaptive responses to low temperature, it was of interest to determine whether *bkd* expression was regulated by temperature. RNA isolated from strain 10403S grown at 37 °C and 10 °C was used to determine the role of low temperature in *bkd* transcription. As shown in Fig. 6(b), there was no significant difference between the *bkd* transcripts at 37 °C and 10 °C, indicating that the transcription of *bkd* remained at the same level when *L. monocytogenes* was grown at 37 °C and 10 °C.

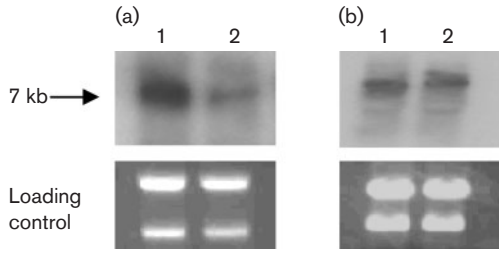

Fig. 6. Northern blot analysis of *bkd* transcription. (a) Total RNA was isolated from *L. monocytogenes* 10403S grown at 37 °C in BHI with the addition of 500 μM α-ketomethylvalerate (lane1), and without addition (lane 2). RNA (10 μg) was hybridized with a ^{32}P-labelled 1·4 kb DNA fragment spanning part of *buk* and *lpd*. (b) Total RNA was isolated from 10403S grown at 37 °C (lane 1) and 10 °C (lane 2). RNA (10 μg) was hybridized with a ^{32}P-labelled 0·4 kb fragment internal to *lpd* locus. The size of the band was determined by comparison with the RNA size marker. rRNA bands were used as a loading control (lower panel).

cld-2 retains its ability to adjust fatty acid composition in response to low temperature when provided with BCFA precursors

As described above, only the precursor for anteiso-BCFAs could restore the growth of *bkd* mutants at low temperature. This was consistent with the observation that the increase in anteiso-$C_{15:0}$ was the most significant fatty acid adaptation to 10 °C in strain 10403S (Table 3) (Annous *et al.*, 1997; Nichols *et al.*, 2002). In addition, the overall fatty acyl-chain length was shorter at 10 °C than at 37 °C (Table 3). These similar adjustments of fatty acid composition could be accomplished in *cld-2*, when the medium was supplemented with either MB or a mixture of MB, IB and IV. Specifically, anteiso-$C_{15:0}$ in *cld-2* was greatly increased from 48 % to 70 % at 10 °C compared to 37 °C when the strain was grown in the presence of MB (Table 3). Therefore, *cld-2* retained the ability to increase the anteiso-$C_{15:0}$ content, and shorten the overall fatty acyl-chain length, when grown at low temperature, which were critical responses for cold adaptation. This implies that the Bkd is not the major cold-regulation point of fatty acid synthesis, and that this regulation probably occurs downstream of Bkd in the fatty acid synthesis pathway.

DISCUSSION

One reason that *L. monocytogenes* is a continuing food safety concern is its ability to grow at refrigeration temperatures. In this report, two Tn*917*-induced mutants showed deficiency in membrane BCFAs, and grew slowly at 37 °C and exceedingly slowly at low temperatures. DNA sequence homology suggested that Tn*917* had inserted into the *bkd* cluster in the two strains. Genetic complementation and supplementation studies demonstrated that the Bkd in *L. monocytogenes* was responsible for the synthesis of branched-chain acyl-CoA derivatives, which are the starting units for BCFA biosynthesis. We also demonstrated the close correlation between membrane BCFA content, especially that of anteiso-$C_{15:0}$, and the growth ability of *L. monocytogenes* at optimal and low temperatures. Although functional Bkd is critical for growth at low temperatures, our data suggest that regulation of the Bkd is not the primary means of cold-regulation of BCFA biosynthesis in *L. monocytogenes*.

Since we sequenced the 5·6 kb *bkd* region of *L. monocytogenes* 10403S, the genome sequences of several *L. monocytogenes* strains have been published (Glaser *et al.*, 2001; Nelson *et al.*, 2004). The *bkd* regions in *L. monocytogenes* strains EGD-e and F2365 are composed of the genes *lmo1369–lmo1374*, and *lmof2365_1386–lmof2365_1391*, respectively. Genetic comparison of the *bkd* regions from the three *L. monocytogenes* strains showed synteny between the strains, with nearly complete identity at the DNA level. Northern blot analysis indicated that the six genes in this cluster (*ptb*,

Table 3. Fatty acid composition of *cld-2* in the presence of short BCFAs in BHI, and of 10403S in BHI, at 37 °C and 10 °C

The values are the means of at least two independent experiments ±SEM; some minor fatty acid components are not shown. ND, Not detected; ECL, equivalent chain-length.

Strain	Temp. (°C)	Percentage (w/w) of total fatty acids										
		$C_{14:0}$	Iso $C_{14:0}$	$C_{15:0}$	Iso-$C_{15:0}$	Anteiso-$_{15:0}$	C1$_{16:0}$	Iso-$_{16:0}$	Iso-$_{17:0}$	Anteiso-$_{17:0}$	BCFA	ECL
10403S	37	ND	0·7±0·1	ND	10·4±0·4	46·2±0·6	0·9±0·1	2·5±0·1	3·4±0·2	35·3±0·4	98·7	15·7
	10	ND	1·1±0·1	ND	12·6±0·3	68·9±1·2	ND	1·4±0·1	0·9±0·1	14·3±0·6	99·3	15·2
cld-2	37*	1·7±0·3	1·7±0·6	1·2±0·3	0·6±0·1	48·4±1·5	7·1±0·7	5·9±1·7	ND	33·1±1·9	89·8	15·7
	10*	3·8±0·6	3·0±0·6	0·6±0·2	0·8±0·1	70·5±2·3	1·9±0·2	3·7±0·8	ND	13·8±0·1	91·9	15·0
cld-2	37†	1·4±0·3	8·5±1·8	0·6±0·1	5·8±0·6	38·0±2·6	4·3±0·1	22·6±2·7	1·3±0·0	17·4±2·6	93·7	15·5
	10†	2·3±0·5	16·3±0·7	0·4±0·0	7·1±0·3	52·7±2·0	1·0±0·4	11·9±0·5	ND	6·2±0·5	94·3	14·8

*Supplemented with 100 μM MB.

†Supplemented with a mixture of 100 μM MB, 100 μM IB and 100 μM IV.

buk, *lpd*, *bkdA1*, *bkdA2* and *bkdB*) were co-transcribed, which was similar to what has been described for *Pseudomonas putida*, *B. subtilis* and *E. faecalis* (Burns *et al.*, 1989; Debarbouille *et al.*, 1999; Ward *et al.*, 2000).

Homology searches also revealed that the Bkd in *L. monocytogenes* exhibited considerable homology to pyruvate dehydrogenase (PDH) from various bacteria (data not shown). PDH and Bkd were the only two members of the 2-keto acid dehydrogenase complex family in *L. monocytogenes* (Glaser *et al.*, 2001) that catalysed the decarboxylation of 2-keto acids, and generated acyl-CoA derivatives. The Bkd mutations in *L. monocytogenes* were not lethal, whereas a *B. subtilis* strain with mutation in both Bkd and PDH required addition of BCFA precursors for growth (Willecke & Pardee, 1971). This suggests that PDH may compensate for the loss of Bkd activity to some extent. Indeed, in *B. subtilis*, PDH showed enzymic activity with branched-chain α-keto acids (Oku & Kaneda, 1988). The PDH enzymic preference (pyruvate > KIV > KMV > KIC) is perfectly consistent with the fatty acid profile in *bkd* mutants, where straight-chain and KIV-derived, even-numbered iso fatty acids rose to compensate for the decreased production of KMV- and KIC-derived odd-numbered fatty acids (Table 1). In addition, KIV was more efficient than KMV and KIC in stimulating growth, and restoring BCFA content (Table 2, Fig. 4). These observations did not match Bkd enzymic preference (KMV > KIV > KIC > pyruvate) (Oku & Kaneda, 1988). The further decrease in BCFA content to 27 % in mutant *cld-2* via pyruvate was presumably because pyruvate competed with branched-chain α-keto acids for PDH. A level of 27 % BCFA might be close to the minimum BCFA necessary for growth of *L. monocytogenes*, in that further increases of pyruvate did not further decrease the BCFA in *cld-2*. Similarly, *B. subtilis* must have a minimum of 28 % BCFAs in the membrane in order to survive (Kaneda, 1991), and *E. coli* needs at least 20 % unsaturated fatty acids (Nunn *et al.*, 1983).

Northern blot analysis showed that transcription of the *bkd* operon was not elevated by low temperature. This was consistent with the observation that total BCFA content was largely unchanged regardless of growth temperatures (Table 3). The major change in fatty acid composition was the increase of anteiso $C_{15:0}$ when *L. monocytogenes* was grown at low temperatures compared to 37 °C. The mechanism of cold-regulated synthesis of BCFAs remains elusive even in *B. subtilis* (de Mendoza *et al.*, 2002; Lu *et al.*, 2004). Strain *cld-2* was able to accomplish a similar adjustment of fatty acid composition in response to low temperatures when BCFA precursors were provided. It seems that cold-regulated shortening of BCFA, in the presence of precursors, is not significantly impaired by the Bkd defect, indicating that Bkd is probably not the major cold-regulation point of fatty acid biosynthesis. Since incorporation of methyl-branches in BCFA could only be achieved by *de novo* biosynthesis of fatty acids (Suutari & Laakso, 1994), the major cold-regulation point is likely to be located downstream of Bkd in the fatty acid biosynthesis pathway.

The substrate preferences of Bkd are involved in determining the ultimate cellular BCFAs, in that the precursor pool produced by Bkd is one major determinant in this pathway (Kaneda, 1991; Willecke & Pardee, 1971). The predominance of anteiso fatty acids in *L. monocytogenes* implies that, in this organism, Bkd has a higher affinity for isoleucine-derived substrates. Impaired Bkd could no longer preferentially produce anteiso precursors, and the fatty acid composition in *cld-2* was largely dependent on the exogenous precursor pool (Table 3). Compared to 10403S (81 %), the decreased total anteiso fatty acid content in *cld-2* (55 %) in the presence of a mixture of MB, IB and IV suggests that preference for anteiso precursors is partially lost due to the Bkd defect (Table 3). Therefore the role of Bkd in preferential synthesis of anteiso precursor may make Bkd essential for growth of *L. monocytogenes* at low temperatures.

The activities of MB, IB and IV in stimulating growth of Bkd mutants at 37 °C were also reported in *B. subtilis* and *Myxococcus xanthus* (Willecke & Pardee, 1971; Toal *et al.*, 1995). Short BCFAs were activated to their acyl-CoA esters by acyl-CoA synthetase, and therefore compensated for the deficiency of BCFA precursors (Rock & Jackowski, 1985) (Fig. 1). Nevertheless, only the anteiso-BCFA precursor MB stimulated growth of Bkd mutants at 10 °C. The varying ability of BCFA precursors to support growth at different temperatures probably reflected the membrane fluidity requirements at that particular temperature. Although both iso-BCFAs and anteiso-BCFAs could provide sufficient membrane fluidity at 37 °C, only anteiso-$C_{15:0}$ could provide the proper fluidity at low temperatures (Edgcomb *et al.*, 2000; Jones *et al.*, 2002). As a result, a substantial amount of anteiso-$C_{15:0}$ was necessary for growth of *L. monocytogenes* at low temperatures. Supplementation with IB and IV led to increased iso-BCFAs at the expense of anteiso-BCFAs. The inhibitory activity of IB and IV on the growth of *cld-2* in the presence of MB at low temperatures was probably due to competitive inhibition of enzymes in the fatty acid biosynthesis pathway, including the initiation enzyme FabH. Preference of these enzymes for isoleucine-derived substrates might explain why only large excesses of IB and IV showed activity. In conclusion, introduction of iso-BCFA-related metabolites should decrease anteiso-BCFA synthesis, and therefore depress or delay the growth at low temperature. This finding has the potential to be used to control the growth of *L. monocytogenes*, specifically at refrigeration temperatures.

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